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厦门大学

博士学位论文

对虾白斑综合征病毒(White Spot Syndrome Virus, WSSV)入胞途径及其分子调控机制研究

Entry and Molecular Regulation of White Spot Syndrome Virus (WSSV) into Crayfish Hpt cell

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摘要

白斑综合征病毒(White spot syndrome virus，WSSV)是对虾的主要病原，该病毒致死率高、传染性强、宿主范围广，是水生甲壳类动物中最为严重的病毒性病原之一。因此，加深对 WSSV 感染致病机理的理解，寻找高效抗病毒防治方法，意义重大。培养细胞是病毒感染研究的有力工具，在甲壳动物原代细胞培养研究中，螯虾造血组织干细胞(Haematopoietic tissue stem cell，Hpt cell)—Hpt 细胞培养较为稳定，是 WSSV 感染研究的首选。本研究以红螯螯虾(*Cherax quadricarinatus*)为研究对象，参照 Söderhäll 等的研究方法培养红螯螯虾 Hpt 细胞，分析 WSSV 在 Hpt 细胞中的感染特性，筛选 Hpt 细胞感染 WSSV 后的差异表达基因，从分子生物学和细胞生物学角度探讨 WSSV 入胞及其分子调控机制以及宿主细胞抵御 WSSV 感染机制。

利用分子生物学技术检测 WSSV 基因表达，结合共聚焦显微镜分析 WSSV 感染 Hpt 细胞的过程，结果显示 WSSV 可在 15 min 内粘附或进入 Hpt 细胞，其中可能涉及病毒囊膜与细胞膜融合入胞过程；感染后 1 h 和 3 h，IE1 和 VP28 分别开始转录表达，表达量持续升高至 96 hpi。此外，单病毒粒子成像研究揭示 WSSV 感染细胞仅占总 Hpt 细胞的 10-15%，提示 WSSV 对不同类型 Hpt 细胞可能具有不同嗜性。

为探究 WSSV 感染 Hpt 细胞分子机制，我们采用抑制性消减杂交技术(Suppression subtractive hybridization，SSH)分析红螯螯虾 Hpt 细胞感染 WSSV 后不同时间(感染极早期 1 hpi 和感染晚期 12 hpi)的基因差异表达。经点杂交筛选、序列测定及生物信息学分析共筛选获得了 366 个病毒感染相关差异表达基因，其中包括网格蛋白轻链基因(Cq-CLC)和伽马氨基丁酸受体相关蛋白(Cq-GABARAP)，为深入研究内吞和自噬等相关活动在 WSSV 入侵宿主细胞中的作用奠定基础。

细胞内吞作用是多数病毒侵入宿主细胞的主要途径。为研究 WSSV 入侵 Hpt 细胞的途径，我们利用透射电镜观察 WSSV 入胞过程，结果显示 WSSV 可以利用网格蛋白介导的内吞(Clathrin-mediated endocytosis，CME)和巨胞饮入侵 Hpt 细胞；进一步研究发现，利用 CPZ 和 dynasore 抑制 CME 可以明显抑制 WSSV

的入胞和复制，利用 EIPA 和 rottlerin 抑制巨胞饮、利用 MβCD 和 filipin 抑制膜穴样凹陷介导的内吞也起到了类似作用，说明 WSSV 可以利用上述三种内吞方式进入宿主细胞。进一步利用 RNAi 技术抑制 Cq-CLC 和 Cq-AP50 等参与 CME 过程的关键因子，揭示 CME 是 WSSV 入侵宿主细胞的关键途径。

克隆获得 Cq-GABARAP 的 cDNA 序列，进一步探讨其介导的 WSSV 入胞机制。RNAi 或添加重组表达 Cq-GABARAP 蛋白后病毒感染试验表明 Cq-GABARAP 可以促进 WSSV 入胞，CPZ 阻断 CME 下游通路能够抑制 Cq-GABARAP 的促进作用，表明 Cq-GABARAP 可能通过调节 CME 上游信号通路以促进 WSSV 入胞；此外，蛋白互作试验发现，Cq-GABARAP 可与包括 VP28 在内的多个 WSSV 囊膜蛋白结合，提示 Cq-GABARAP 与病毒粒子直接结合可能是其促进 WSSV 入胞的另一机制；电镜观察发现，病毒感染早期 WSSV 可与自噬小体共定位，自噬调节制剂改变了 Hpt 细胞自噬活性、进而影响了 WSSV 入侵 Hpt 细胞，表明自噬可能参与内吞活动从而调节 WSSV 入胞。

此外，本研究还发现 Cq-GABARAP 具有抗 WSSV 功能。基因表达差异分析显示 Hpt 细胞中 Cq-GABARAP 在感染 WSSV 后表达上调，RNAi 或重组表达蛋白孵育后感染病毒试验揭示 Cq-GABARAP 是一个抗 WSSV 因子，免疫印迹和电镜观察试验揭示 Cq-GABARAP 可促进 Hpt 细胞摄入 WSSV，并汇聚入胞病毒粒子成团，抑制病毒粒子入核启动复制，从而发挥抗 WSSV 作用；雷帕霉素诱导自噬活性增强后，Hpt 细胞内 WSSV 复制受到抑制，说明自噬可能是一种重要的抗 WSSV 防御反应。

综上，本研究为 WSSV 感染入胞及其调控机制研究提供了重要信息，并为抗 WSSV 靶点药物研发提供重要理论基础。

关键词：白斑综合征病毒；红螯螯虾；Hpt 细胞；内吞；伽马氨基丁酸受体相关蛋白

Abstract

WSSV is a lethal pathogen of penaeus shrimp, and also become one of the most vital viral pathogen for many aquatic crustacean for its nature of high lethality, high contagiosity and wide range of host. It is urgent to develop effective antiviral strategies which require a better understanding of the pathogenesis of WSSV. It has been well reported that crayfish haematopoietic tissue stem cell (Hpt cell) is one of the most successful primary cell culture in crustacean for WSSV infection study. In this study, we cultured Hpt cell from *Cherax quadricarinatus* as described by Söderhäll et al, isolated differentially expressed genes from Hpt cell infected with WSSV and explored cellular entry of WSSV as well as its regulation mechanism and the antiviral immune reaction of host cell mediated by Cq-GABARAP at the molecular and cellular level.

To characterize the WSSV infection process, we combined molecular biological methods and confocal microscopy to determine WSSV genes expression and to conduct single virus image study, respectively. The data indicated WSSV could attach and/or enter Hpt cell within 15 min, and progress into a replication cascade, as shown by the initiation expression of IE1 and VP28 at 1 hpi and 3 hpi, respectively. The expression level of virus genes increased during the whole recorded period till 96hpi. Our data also suggested WSSV entered Hpt cell in a successive manner, which might involve a mechanism mediated by fusion between envelope membrane and plasma membrane. Besides, only 10-15% of Hpt cell were recorded positive of DiD-WSSV, which indicated WSSV had limited tropism to certain types of Hpt cell.

To gain novel insight of into the key biological process during WSSV infection, we chose red claw crayfish Hpt cell infected with WSSV at early and late stage, and used suppression subtractive hybridization (SSH) to elucidate the cellular response to WSSV challenge at the transcriptional level. After screening by dot blotting, sequence determination and bioinformatic analysis, 366 novel genes were isolated to be transcriptionally involved in WSSV infection. Among these genes, Cq-CLC and

Cq-GABARAP were two important genes, which will benefit the study of endocytosis and autophagy as well as their roles in WSSV infection in red claw crayfish.

Endocytosis, a process used by cell to take up extracellular material, is employed by viruses for internalization. To explore how WSSV enter Hpt cell, transmission electron microscopy (TEM) was used to visualize the infection. Under TEM, we visulized WSSV might enter Hpt cell via clathrin-mediated endocytosis (CME) and macropinocytosis. Later, pharmacological inhibitors was used to dissect the entry routes by block corresponding endocytosis pathway. Pretreatment of Hpt cell with CPZ or dynasore to block CME resulted in dramatic decrease in WSSV entry as well as replication. Similar results were obtained when interupting macropinocytosis with EIPA or rottlerin, or disturbing caveolae-mediated endocytosis with MβCD or filipin. Our data strongly suggested CME, macropinocytosis and caveolae-mediated endocytosis may be employed by WSSV to enter host cell. Furthermore, knock down of Cq-CLC or Cq-AP50, key components of CME, significantly interfered the entry and replication of WSSV in Hpt cell, which confirmed CME as an important portal for WSSV internalization into Hpt cell.

After gene cloning of Cq-GABARAP, the mechanism of WSSV internalization mediated by Cq-GABARAP was explored. The assays of RNAi or recombinant Cq-GABARAP (rCq-GABARAP) incubation followed by WSSV infection revealed Cq-GABARAP had a role in affecting WSSV entry. CPZ treatment canceled the WSSV uptake promoted by Cq-GABARAP, suggesting Cq-GABARAP acted as upstream regulator of CME. Protein interaction study shown Cq-GABARAP could bind to more than five envelope proteins, including VP28, which supported the notion that Cq-GABARAP may promote WSSV entry by carrying virions. WSSV were found to locate to autophagosomes under TEM at early stage during infection. Furthermore, WSSV internalization was altered when autophagy modulators was used to adjust autophagy process. These data indicated autophagy could be another potential mechanism of WSSV entry mediated by Cq-GABARAP.

Besides, the anti-WSSV function of Cq-GABARAP was characterized.

Upregulation of Cq-GABARAP in Hpt cell infected with WSSV implied Cq-GABARAP functioned in host response against WSSV. Later, Cq-GABARAP was found to act as an antiviral factor by Cq-GABARAP RNAi or rCq-GABARAP incubation assay. Both results from western blotting and TEM shown WSSV virions accumulated in Hpt cell when treated with rCq-GABARAP, suggesting rCq-GABARAP may inhibit WSSV replication by redirecting WSSV into a non-productive pathway. Moreover, WSSV gene expression was suppressed by autophagy induction with rapamycin. The data suggested autophagy may be an important anti-WSSV defense reaction.

In summary, the study here expanded our knowledge of the pathogenesis of WSSV as well as the molecular regulation of WSSV entry into Hpt cell, which will benefit the development of anti-WSSV strategy.

Key Words: White spot syndrome virus (WSSV); *Cherax quandricarinatus*; Haematopoietic tissue stem cell (Hpt cell); Endocytosis; Cq-GABARAP

缩略词中英文对照表

英文缩写	英文全称	中文全称
AP50	Adaptor protein complex mu subunit	衔接蛋白复合体中亚基
Atg8	Autophagy-related gene 8	自噬相关基因 8
bp	Base pair	碱基对
cDNA	Complementary DNA	互补脱氧核糖核酸
CLC	Clathrin light chain	网格蛋白轻链
CME	Clathrin-mediated endocytosis	网格蛋白介导的内吞
CPBS	Crayfish phosphate buffer saline	螯虾磷酸盐缓冲液
Ct	Cycle threshold	阈值循环数
DAPI	4’, 6-diamidino-2-phenylindole	4，6-二脒基-2-苯基吲哚
DiD	1,1'-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate	1，1'-双十八烷基-3，3，3’，3’-四甲基吲哚二碳菁高氯酸盐
dsRNA	Double strands RNA	双链 RNA
EB	Ethidium bromide	溴化乙啶
FDA	Fluorescein diacetate	二乙酰荧光素
GABARAP	γ-aminobutyric acid receptor-associated protein	伽马氨基丁酸受体相关蛋白
GST	Glutathione S-transferase	谷胱甘肽-S 转移酶
hpi	Hours post infection	感染后几个小时
Hpt	Haematopoietic tissue	造血组织
kDa	Kilodalton	千道尔顿
MOI	Multiple of infection	感染复数
mRNA	Messenger RNA	信使 RNA
ORF	Open reading frame	开放阅读框
PBS	Phosphate buffer saline	磷酸盐缓冲液
PCR	Polymerase chain reaction	聚合酶链式反应
PI	Propidium iodide	碘化丙啶

续表

英文缩写	英文全称	中文全称
RACE	Rapid amplification of cDNA ends	cDNA 末端快速扩增技术
Rapa	Rapamycin	雷帕霉素
SDS-PAGE	SDS-polyacrylamide gel electrophoresis	SDS-聚丙烯酰胺凝胶电泳
TEM	Transmission electron microscopy	透射电镜
WB	Western blotting	蛋白印记